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Changing kinetic properties of the two-enzyme phosphoglycerate kinase / NADP-linked glyceraldehyde-3-phosphate dehydrogenase couple from pea chloroplasts during photosynthetic induction

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The kinetics of the two enzyme phosphoglycerate kinase (EC 2.7.2.3) / NADP-linked glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13) couple are negatively cooperative and will also fit a model for two enzymes acting on one substrate. When the chloroplast is illuminated apparent negative cooperativity is reduced; maximal velocity of only one of the two enzymes in the two-enzyme model is increased. Even after light activation the activity of glyceraldehyde-3-phosphate dehydrogenase appears to be too low to support photosynthesis at calculated levels of glycerate-1,3-bisphosphate in isolated chloroplasts (Marques, I.A., Ford, D.M., Muschinek, G. and Anderson, L.E. (1987) Arch. Biochem. Biophys. 252, 458–466). The activity of the coupled reaction is apparently sufficient to support observed rates of CO_2 fixation, which suggests that glycerate-1,3-bisphosphate may be channeled from the kinase to the dehydrogenase in vivo.

Introduction

Before illumination reductive pentose phosphate cycle enzyme activity levels in isolated chloroplasts are low. This is because four of these enzymes are dark inactivated and also because substrate levels are low [1–4]. When the chloroplast is illuminated, enzyme activation and the ensuing increase in substrate levels leads to an acceleration of CO_2 fixation until steady state photosynthetic rates are attained [5].

We now report that the kinetics of the two enzyme coupled reaction catalyzed by phosphoglycerate kinase (EC 2.7.2.3) and the dark form of NADP-linked glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13) are multiphasic when phosphoglycerate is the varied substrate. When the dehydrogenase is light activated, activity at physiological phosphoglycerate levels increases and the deviation from normal Michaelis–Menten kinetics is markedly diminished. The kinetics observed are consistent with, but do not prove, channeling of bisphosphoglycerate from phosphoglycerate kinase to glyceraldehyde-3-phosphate dehydrogenase in the chloroplast.

Materials and Methods

Isolation of chloroplasts and measurement of O_2 evolution. Pea (*Pisum sativum* L. var Little Marvel) plants were grown and chloroplasts isolated from (10–11)-day-old seedlings as described previously

Abbreviations: Hepes, 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol.

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[6], except that the plants were grown in Sunshine seedling mix no. 3. O_2 evolution was followed using a Clark-type O_2 electrode as described previously [6].

Measurement of NADP-linked glyceraldehyde-3-phosphate dehydrogenase activity. In experiments in which the kinetics of the light and dark forms of the kinase-dehydrogenase couple were analyzed in the Calvin cycle direction, 0.5 ml aliquots were removed from the O_2 evolution mixture into 4.5 ml ice-cold distilled H_2O. After centrifugation (27000 × *g*, 15 min) 100 µl aliquots (equivalent to 1 µg chlorophyll in the O_2 evolution assay) of the supernatant solution were used for assay of enzyme activity. The standard reaction mixture was (final concentrations in 1 ml, pH 7.8) 50 mM Hepes (K^+), 10 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 2.5 mM ATP, 0.2 mM NADPH or NADH, 5 mM phosphoglycerate and (from the extract) 3.3 mM sorbitol, 0.1 mM $NaHCO_3$, 50 µM $Na_4P_2O_7$, 2 µM K_2HPO_4, and 2 units catalase. The concentration of one substrate was varied. Phosphoglycerate concentrations were varied by 17 even logarithmic intervals from 0.01 to 20 mM. When ATP was varied the concentrations used were 0.03, 0.06, 0.09, 0.15, 0.3, 0.6, 1.5 or 2.5 mM. When NADH was varied the concentrations used were 0.06, 0.12, 0.18, 0.24, 0.3, 0.4 or 0.6 mM. When NADPH was varied the concentrations used were 0.004, 0.008, 0.012, 0.016, 0.02, 0.04, 0.1 and 0.2 mM. Reaction was followed on a Varian Cary 210 or 219 recording spectrophotometer. Assay temperature was 23° C. Extraneous phosphoglycerate kinase was not added to the assay cuvette.

Estimation of kinetic parameters. The kinetics were analyzed using the computer program of Hanson et al. [7] on an IBM 3081K64 computer at the University of Illinois, Chicago. Velocity values used were the steady-state values. Where biphasic kinetics were observed, the data were analyzed in sections. Data points in the transition areas were not included in the analysis. Hill coefficients were estimated graphically. Weighted mean values for K_m and standard error were estimated using the reciprocal of the variance as the weighting factor.

Chlorophyll. Chlorophyll was determined by the method of Arnon [8].

Chemicals. Biochemicals were obtained from Sigma Chemical Company. All other reagents were of analytical reagent grade or of the highest quality commercially available. Pea seeds were obtained from Northrup and King, Chicago, IL. Sunshine seedling mix no. 3 was obtained from Fisons-Western Co., Vancouver, BC, Canada.

Results

We found that the activity of the phosphoglycerate kinase/glyceraldehyde-3-phosphate dehydrogenase couple is modified when intact chloroplasts are irradiated (data not shown). Activation is observed if NADPH is the reducing substrate, but activity is transiently affected and finally slightly lower when NADH is the reducing substrate. Similar results have been reported earlier for the dehydrogenase [5,9].

The kinetics of the two enzyme phosphoglycerate kinase/NADP-linked glyceraldehyde-3-phosphate dehydrogenase couple are multiphasic, when NADP-linked glyceraldehyde-3-phosphate dehydrogenase is in the dark (less active) form and NADPH is the reducing substrate and glycerate-3-phosphate the varied substrate (Fig. 1). When NADH is the reducing substrate apparent one phase kinetics are observed (Fig. 2). Multiphasic kinetics have been reported for the DTT-activated

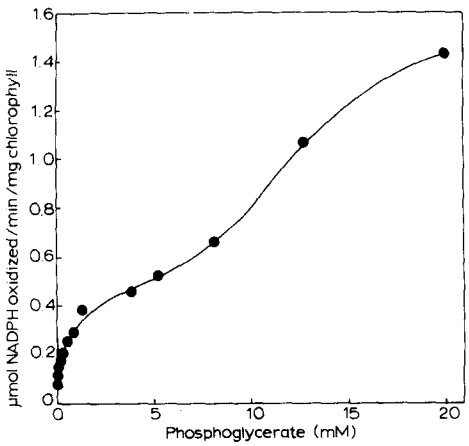

Fig. 1. NADPH-dependent activity of the phosphoglycerate kinase/glyceraldehyde-3-phosphate dehydrogenase couple as a function of 3-phosphate-glycerate concentration. The dehydrogenase has not been light activated. Similar results were obtained in six experiments. In one experiment the kinetics were monophasic. For experimental details see Materials and Methods.

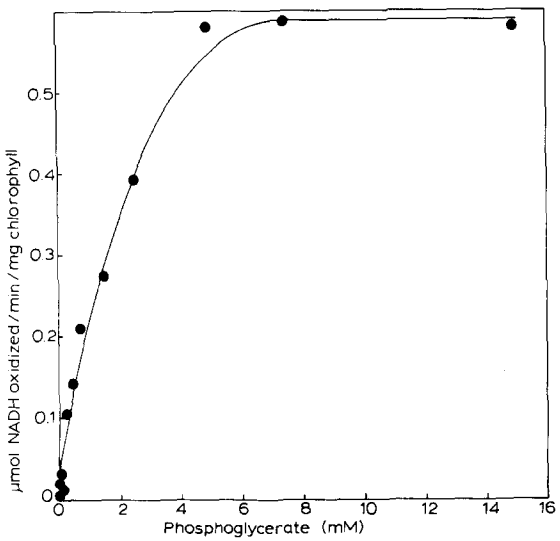

Fig. 2. NADH-dependent activity of the phosphoglycerate kinase/glyceraldehyde-3-phosphate dehydrogenase couple as a function of 3-phosphate-glycerate concentration. The dehydrogenase has not been light activated. Although there is no evident cooperativity, the data do not fit well to a rectangular hyperbola. Apparent monophasic kinetics were found in four experiments. In two experiments apparent biphasic kinetics were observed.

enzyme in spinach extracts [10]. These kinetics can be analyzed as three separate phases: (1) phosphoglycerate levels below 0.8 mM; (2) phosphoglycerate concentrations from 0.8 to 5 mM; (3) levels of phosphoglycerate above 5 mM. K_m and V_{max} value estimates for the intermediate levels of phosphoglycerate are shown in Figs. 3 and 4. With high levels of phosphoglycerate (5–20 mM) $K_{m\,phosphoglycerate}$ was between 5 and 6 mM. With the low phosphoglycerate concentrations the variance in the K_m estimates was quite large. The variance in the K_m and V_{max} estimates is greater if the data are analyzed as only two phases.

The response of the two enzyme couple to phosphoglycerate concentration is a function of the time of illumination of the chloroplast when NADPH is the reducing substrate (Figs. 3–5). During light activation the apparent $K_{m\,phosphoglycerate}$ at the intermediate phosphoglycerate levels decreases to a minimum value and then increases to a value slightly higher or the same as the initial K_m (Fig. 3). At high phosphoglycerate levels the aparent $K_{m\,phosphoglycerate}$

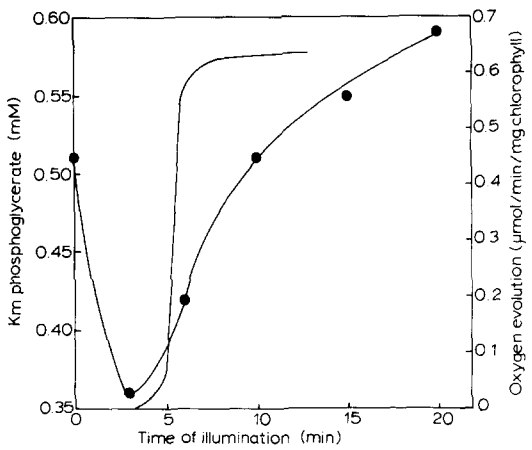

Fig. 3. $K_{m\,phosphoglycerate}$ values for phosphoglycerate kinase/NADP-linked glyceraldehyde-3-phosphate dehydrogenase as a function of time of illumination when phosphoglycerate is varied from 0.5 to 5 mM. Rate of O_2 evolution (solid line without data points) is shown in background. Standard error was less than 10% for each K_m value. Similar results were obtained in two additional experiments.

decreases about 10-fold during light activation (data not shown). When NADH is the reducing substrate the response to light activation is minimal (data not shown).

These kinetics could be indicative of (1) the biphasic kinetics of phosphoglycerate kianse with

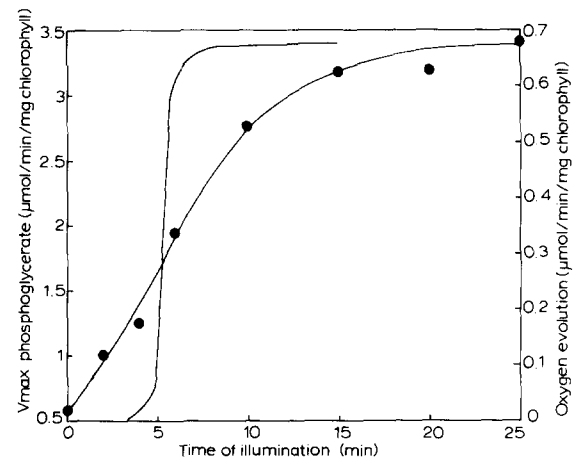

Fig. 4. V_{max} values for phosphoglycerate kinase/NADP-linked glyceraldehyde-3-phosphate dehydrogenase as a function of time of illumination. Standard error was less than 10% for each V_{max} value. Rate of O_2 evolution (solid line without data points) is shown in background.

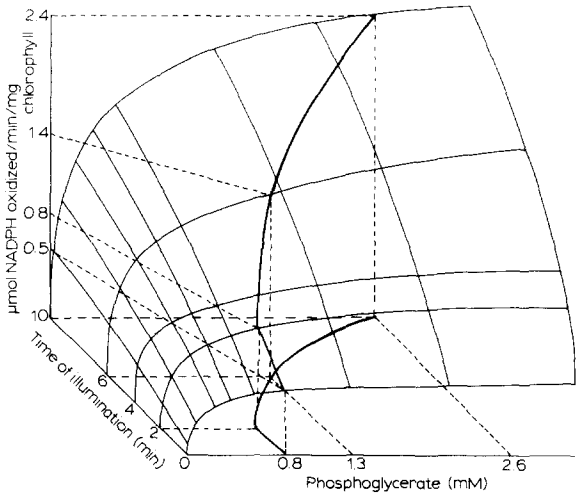

Fig. 5. Tridimensional plot of v_0 for the coupled kinase/dehydrogenase reaction vs. phosphoglycerate concentration and time of illumination. Upper heavy line shows predicted activity if phosphoglycerate levels are those found by Marques et al. [5] (lower heavy line). Phosphoglycerate levels below 6 min are extrapolated from data in Ref. 6. The data were from experiment in Fig. 4. Activity levels in vivo would be about 20% lower, assuming that ATP levels are 1.2 mM [19] rather than 2.5 mM (used in the assay cuvette) and ignoring ADP inhibition.

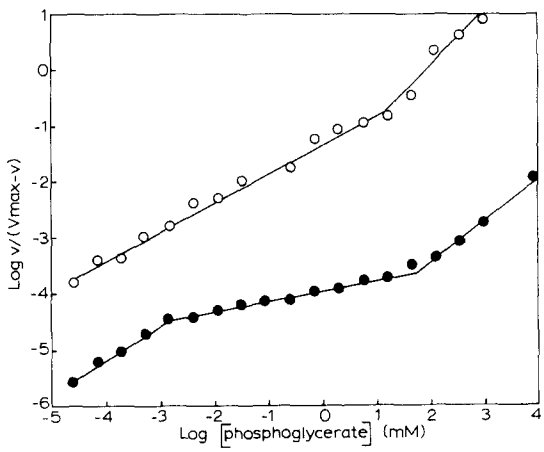

Fig. 6. Hill plot for phosphoglycerate kinase/NADP-linked glyceraldehyde-3-phosphate dehydrogenase activity with phosphoglycerate as the varied substrate. Extract from dark-treated chloroplasts (●) and from chloroplasts illuminated for 25 min (○). The data were from experiment in Fig. 4. Similar results were found in nine experiments. Normal Michaelis–Menten kinetics were found in one experiment.

respect to phosphoglycerate [11], (2) negative cooperativity which is more pronounced when NADPH is bound to glyceraldehyde-3-phosphate dehydrogenase, or (3) the presence of two glyceraldehyde-3-phosphate dehydrogenases in the extract. It seems unlikely that the biphasic kinetics of the kinase (possibility 1) are responsible for the triphasic kinetics observed here, because the kinetic constants are not those of the chloroplastic phosphoglycerate kinase (compare values reported here and those in Ref. 11). In addition, the pyridine nucleotide substrate and the activation state of the dehydrogenase clearly affect the kinetics of the two-enzyme couple. If the kinase were rate limiting, then the effect of the dehydrogenase should be nil. Our experiments are equally consistent with possibilities (2) and (3). On a Hill plot the kinetics are apparently negatively cooperative when NADPH is the reducing substrate (Fig. 6). Negative cooperativity decreases with irradiation (Fig. 7) and is virtually eliminated when NADH is the reducing substrate (Fig. 2). The kinetics with NADPH as the reducing substrate also fit the two-enzyme model of Spears et al. [12], which would apply to two forms of one enzyme (Fig. 8). There is no indication that light activation shifts one form to the other. Wolosiuk and Buchanan [13] also found evidence for more than one form of spinach NADP-linked glyceraldehyde-3-phos-

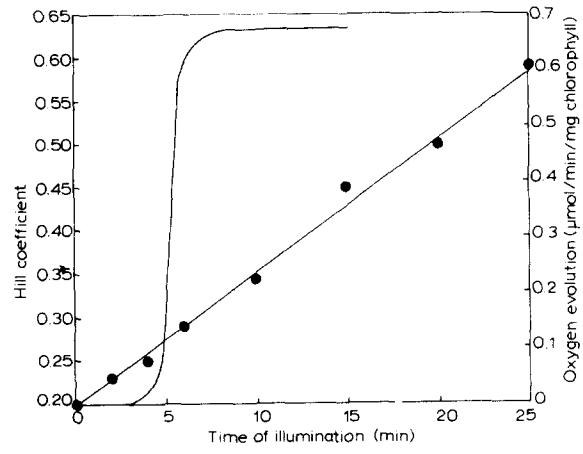

Fig. 7. Hill coefficient vs. time of illumination for phosphoglycerate kinase/NADP-linked glyceraldehyde-3-phosphate dehydrogenase activity. The rate of O_2 evolution (solid line without data points) is shown in the background. The data were from experiment in Fig. 4. Similar results were obtained in a duplicate experiment.

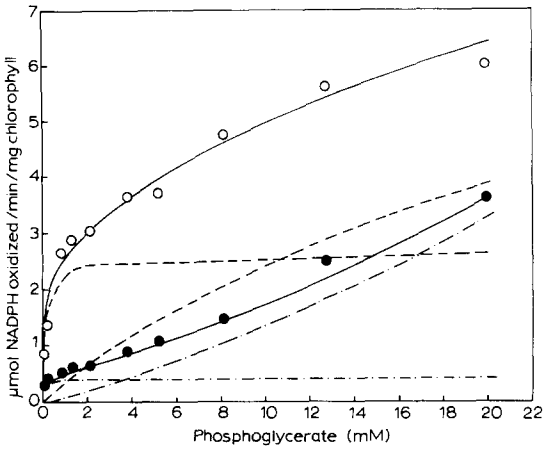

Fig. 8. Activity of the two enzyme phosphoglycerate kinase, NADP-linked glyceraldehyde-3-phosphate dehydrogenase reaction analyzed as two enzymes acting on one substrate. The solid line is the sum of activity of both 'enzymes'. Data points are experimentally obtained values in light (○) or dark (●). Maximal velocity values for 'Enzyme 1' increase with illumination while maximal velocity values for 'Enzyme 2' decrease. $K_{m\,phosphoglycerate}$ for 'Enzyme 1' is 0.03 mM before illumination and 0.1 mM after illumination. $K_{m\,phosphoglycerate}$ for 'Enzyme 2' is 30 mM after illumination. The kinetics of 'Enzyme 2' are Michaelis–Menten only after the chloroplast is illuminated. Similar results were found in a duplicate experiment.

phate dehydrogenase, only one of which could be light-activated. The light-dependent change in electrophoretic mobility reported at this laboratory [14] could also represent activation of only one form of the dehydrogenase. Further examination of the kinetics with purified glyceraldehyde-3-phosphate dehydrogenase will clearly be necessary before we can distinguish between negative cooperativity and two forms of the enzyme competing for bisphosphoglycerate.

TABLE I

K_m VALUES FOR THE PHOSPHOGLYCERATE KINASE/GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE COUPLE BEFORE AND AFTER LIGHT ACTIVATION OF THE DEHYDROGENASE

The number in parenthesis is the number of separate experimental determinations.

Varied substrate	K_m (mM)		Effect of light modulation on V_{max}
	Dark Form	Light Form	
NADPH	0.032 ± 0.002 (2)	0.030 ± 0.001 (2)	Increase
NADH	0.19 ± 0.01 (4)	0.45 ± 0.02 (4)	No effect
ATP [a]	0.18 ± 0.02 (4)	0.37 ± 0.02 (4)	Increase

[a] NADPH was the pyridine nucleotide substrate and the $MgCl_2$ concentration was 5 mM. K_m was the same when higher (up to 15 mM) levels of $MgCl_2$ were present in the assay mixture.

K_m values for the light-activated and dark forms of the dehydrogenase/kinase couple for ATP, NADPH and NADH are given in Table I. These values are in the range of those reported by other workers and in this laboratory [11,15,16].

Estimates of the activity of the two enzyme kinase/dehydrogenase reaction as a function of phosphoglycerate concentration and time of illumination are shown in Fig. 5. Notably, the simulated activity of the two-enzyme couple in Fig. 5 always exceeds the activity required to account for O_2 evolution (trace shown in Fig. 4). At 6 min the rates are about equal. At 10 min the potential kinase/dehydrogenase activity is twice the rate of O_2 evolution. In previous experiments with the pea chloroplast system $^{14}CO_2$ fixation and O_2 evolution were found to be parallel [5].

Discussion

Although the NADP-linked stromal glyceraldehyde-3-phosphate dehydrogenase uses either pyridine nucleotide [17], the light activation of the dehydrogenase observed in these experiments is dependent on the pyridine nucleotide in the assay. Because the deviation from Michaelis–Menten kinetics is much more pronounced when NADPH is the reducing substrate and the varied substrate is phosphoglycerate, our results suggest that the bound pyridine nucleotide affects the carbon substrate binding site, altering the interaction of the enzyme with that substrate. The light-dependent change in activity is probably the result of reduction of a disulfide bond followed by a change in conformation [4]. The light-induced change in conformation apparently produces further effects which are most pronounced in the Calvin cycle direction when NADPH is the reducing substrate.

The physiological substrate for the NAD-linked glyceraldehyde-3-phosphate dehydrogenases is probably the phosphoglycerate kinase-bisphosphate-glycerate complex [18]. Phosphoglycerate

promotes glyceraldehyde-3-phosphate formation in the halibut system [18]. The apparent negative cooperativity observed here may be related to this effect. In experiments in which substrate levels and enzyme activities in intact photosynthesizing chloroplasts were estimated, the activity of glyceraldehyde-3-phosphate dehydrogenase appeared to be insufficient to support photosynthetic CO_2 fixation [5]. Activity levels for the kinase/dehydrogenase complex appear to be sufficient to support CO_2 fixation both during induction and when steady-state photosynthesis is reached (Figs. 4 and 5). Clearly light activation of the dehydrogenase is necessary for photosynthetic CO_2 fixation. These data also suggest that bisphosphoglycerate is transferred directly from phosphoglycerate kinase to glyceraldehyde-3-phosphate dehydrogenase in the intact chloroplast.

We are currently examining the kinetics of the two-enzyme reaction with the purified stromal enzymes with the aim of establishing that bisphosphoglycerate is channeled from phosphoglycerate kinase to glyceraldehyde-3-phosphate dehydrogenase in the chloroplast.

Acknowledgements

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